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IMB-SD62, a triazolothiadiazoles derivative with promising action against tuberculosis

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ABSTRACT

One lead 3,6-disubstituted 1,2,4-triazolo[3,4-b][1,3,4]thiadiazole was identified as an inhibitor of shikimate dehydrogenase with antitubercular activity. Following up this compound, we optimized the lead through systematic modification of the 3 and 6 positions. The antitubercular activities *in vitro*, shikimate dehydrogenase inhibitory activities and cytotoxicity of derivatives were determined. We found IMB-SD62 with lower cytotoxicity and better activity. Thus, we studied the *in vivo* efficacy of IMB-SD62 against *Mycobacterium tuberculosis* and pharmacokinetics of IMB-SD62. *In vivo* acute *M. tuberculosis* H37Rv infection assay, IMB-SD62 showed antitubercular activity with the mean lung CFU counts decreasing 1.7 lg. The plasma pharmacokinetics study in rats showed that the oral bioavailability of IMB-SD62 was 14% and the half time was 1.05 h. The results of tissue distribution indicated that IMB-SD62 was mainly absorbed by liver and lung. *In vitro* metabolism study suggested that the metabolic ways of IMB-SD62 were dealkylated, oxidized and demethylated. CYP enzyme inhibition of IMB-SD62 in human liver microsomes was also evaluated. IMB-SD62 showed barely inhibition on CYP3A4 and CYP2D6. The excretion study manifested that IMB-SD62 was mainly eliminated by fecal excretion in rats. We concluded that based on these pharmaceutical properties, IMB-SD62 has the potential to be developed into new TB drug.

1. Introduction

Tuberculosis (TB), a chronic infectious disease caused by *Mycobacterium tuberculosis (Mtb)*, still has high morbidity and mortality. According to WHO, there were about 10 million patients who developed into TB and 1 million death every year [1]. The emergence of multidrug-resistant TB (MDR-TB), extensively drug-resistant TB (XDR-TB) and the co-infected with HIV makes the therapy to become a tough task [2]. Unfortunately, the drugs now available are largely responsible for drug-sensitive TB and the period of chemotherapy with these remedies is least 6 months. The treatment regimens for drug-resistant TB include expensive second-line drugs, which have low activity and high toxicity that must be administered by both oral and parenteral routes for up to 24 months [3]. It is extremely urgent to develop new anti-TB drugs with novel modes of action, shorten the treatment regimen or reduce the toxicity of clinical TB drugs.

It has been reported that triazolothiadiazoles to have various functions such as antibacterial [4–6] antifungal [7], antiviral [8], antitumor [9–11], anti-inflammatory [12] and antioxidant [13]. Several drugs including triazole, like flutrox, nefazodone, trazodone, triazoledione, etc., have already been used in clinic. The antitubercular activity of triazolothiadiazole has also been reported [14]. Recently, we have being screening inhibitors of *Mtb* shikimate dehydrogenase (*Mtb* SD) for discovering novel antitubercular drugs. Through screening, One 3,6-disubstituted triazolothiadiazole(*Mtb* SD-IC_{50} of this compound is 23.00 μg/mL) was discovered [28]. The MICs of this compound against $H37R_V$, rifampin resistant strains, isoniazid and rifampin resistant strains were 8.0 μg/mL [15]. However, the lead compound has low water solubility and can easily be metabolized *in vivo*, thus the structural modification of the lead compound has been carried out. Through structure–activity relationships (SAR) study, we optimized the lead through systematic structural modifications at the 3 and 6 positions and finally obtained 100 derivatives [15,16].

In this study, in order to obtain functional enzyme with higher yield, we firstly optimized the condition for expression and purification of *Mtb* SD. Biological activities of derivatives against drug-susceptible and drug-resistant *Mtb* strains, enzyme inhibitory activities of derivatives and cytotoxicity of derivatives were determined. We found IMB-

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Abbreviations

ATCC	American Type Culture Collection
AUC	Area under the concentration-time curve
CMC	Carboxymethylcellulose
CFU	Colony-Forming Units
XDR-TB	Extensively Drug-Resistant TB
$T_{1/2}$	Half-Lifes
HBSS	Hank's Buffered Salt Solution
IV	Intravenous Injection
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	*Luria–Bertani* Medium
MCT	Medium-Chain Triglycerides
MIC	Minimal Inhibitory Concentrations
MDR-TB	Multidrug-Resistant TB
Mtb	*Mycobacterium tuberculosis*
OADC	Oleic Albumin Dextrose Catalase
PBS	Phosphate Buffer Saline
PO	Peros
PAGE	Polyacrylamide Gel Electrophoresis
SD	Shikimate Dehydrogenase
SDS	Sodium Dodecyl Sulfate
Tris	Trihydroxymethylaminomethane
TB	Tuberculosis
IC_{50}	50% Inhibiting Concentration

SD62,3-(4-Bromophenyl)-6-((4-methoxyphenoxy)methyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole, with stronger antitubercular activity, stronger enzyme inhibitory activity and lower cytotoxicity than the lead compound. The MIC of IMB-SD62(named 6d-5 in Ref 15) against H37Rv, rifampin resistant strains, isoniazid and rifampin resistant strains were 2 μg/mL, 4 μg/mL and 8 μg/mL separately [15]. To evaluate the druggability of IMB-SD62, we also determined the antitubercular activity of IMB-SD62 *in vivo* acute *Mtb* infection model. Afterwards, the plasma pharmacokinetics, tissue distribution, *in vitro* metabolism and excretion of IMB-SD62 were thoroughly studied.

2. Materials and methods

2.1. Strains and compounds

E. coli BL21 star™ (DE3) with plasmid pET28a-SD was built and stored in Institute of Medicinal Biotechnology Chinese Academy of Medical Sciences. The *Mtb* strain used in this study was the laboratory strain H37Rv (ATCC 27294; American Type Culture Collection, Rockville, MD). The *E. coli* was cultured in *Luria–Bertani* (LB) broth or on LB agar medium. The *Mtb* was cultured on Middlebrook 7H11 agar media supplemented with OADC or in 7H9 broth plus OADC and polysorbate 80. His trap™ HP Ni-sepharose was from GE Healthcare Biosciences. ÄKTA explore prime was from General Electric Company. BCA protein assay kit was from CW Biotech. Kanamycin was purchased from Amresco. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Merck. Lactose and isoniazid were purchased from Sigma. Triazolothiadiazoles derivatives were synthesized at Institute of Medicinal Biotechnology Chinese Academy of Medical Sciences.

2.2. Expression and purification of recombinant SD

For over-expression, the strain was cultured in LB medium containing kanamycin (the final concentration is 100 μg/mL) at 37 °C to get an optical density at 600 nm (OD_{600}) of 0.6. Then IPTG at a final concentration 20 μmol/L and 6.25% lactose were added and the bacterium was induced at 25 °C for 24 h. Cells were harvested by centrifugation at 4 °C, and then were suspended. The suspension was disrupted by high pressure and the lysate was centrifuged at 8000 g for 30 min at 4 °C. The cell extract was applied to His trap™ HP Ni-sepharose by ÄKTA explorer prime. The bound *Mtb* SD proteins were eluted by washing buffer containing 150 mmol/L imidazole. The purified *Mtb* SD was analyzed by SDS-PAGE and quantified by BCA protein assay kit. The purified *Mtb* SD solution was stored at −80 °C until use.

2.3. Enzyme assay and In vitro biological assays

Mtb SD enzyme can catalyze the substrate shikimic acid to produce 3-dehydroshikimate with $NADP^+$ converted to NADPH. The *Mtb* SD activity was determined by measuring NADPH's absorbance at 340 nm. The enzyme assay was conducted in 96-well plates with a 100 μL reaction volume at 37 °C in the presence of 100 mmol/L Tris HCl, 1 mmol/L shikimic acid, 0.5 mmol/L $NADP^+$ and SD. The incubation time was 60 min and the absorbance at 340 nm was detected once per minute. All reactions were conducted in triplicate.

2.4. In vivo acute Mtb H37Rv infection assay

The protocol was almost the same as previously described [17]. Briefly, SPF BALB/c male mice, weighing 20–22 g, were used in the present study. Mice were infected via aerosol with a suspension of *Mtb* H37Rv. After ten days, isoniazid and IMB-SD62 were dissolved or suspended in 0.5% carboxymethylcellulose (CMC) and administered by oral gavage. The doses of isoniazid and IMB-SD62 were 25 mg/kg and 50 mg/kg separately. Mice received CMC only were the control group. The drugs were given once a day for 15 days. Mice were killed the day after the last day of treatment; the bacterial colonies in lungs were counted. Mean log values were calculated from bacterial burden counts. There were 6 mice in every group.

2.5. Plasma pharmacokinetics

Male Sprague-Dawley rats were used with three rats per time point. When administered through intravenous injection, IMB-SD62 was prepared in DMSO: PEG400: Water = 20:60:20 and the dose was 2 mg/kg. For oral administration, IMB-SD62 was dissolved in medium-chain triglycerides (MCT) and a homogeneous opaque suspension was administrated to achieve a 5 mg/kg dose. Blood samples were collected at 5 min, 15 mim, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h and 24 h after administration. The samples were analyzed by LC-MS/MS. The analytical column was ACQUITY UPLC BEH C18 (2.1 × 50 mm, 1.7 μm). The mobile phase was prepared by acetonitrile and water containing 0.025% fluoroacetic acid and 1 mmol/L NH_4OAC in various proportions. The flow rate was 0.6 mL/min. The MS condition was: ESI positive; SRM detection; IMB-SD62 $[M+H]^{+5}$m/z 417.12/293.9. A non-compartmental library model was used to calculate PK parameters, including the maximum concentration of drug in plasma (Cmax), the elimination half-life($T_{1/2}$), the area under the concentration-time curve ($AUC_{0\text{-last}}$), clearance rate and bioavailability.

2.6. Tissue distribution of IMB-SD62

The rats and analytical method were the same as plasma pharmacokinetics study. Rats were administrated at the dose of 5 mg/kg orally. The samples of lung, liver, spleen, heart, kidney and plasma were collected at 2, 6, 10 and 24 h after administration. There were three rats at every time. The drug concentrations in plasma and tissues were detected and the tissue/plasma concentration ratios of each point were calculated.